CYSTEINE LOSSES RESULTING FROM ACID HYDROLYSIS OF PROTEINS

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In the course of a study of the relation between molecular cross-bonding and the dimensional stability of fibrous proteins, particularly the keratins, it was necessary to determine small amounts of cysteine, about 1 per cent of the sample. For this purpose, a method used by a number of workers was adopted. This method consisted in hydrolysis of the protein by refluxing it with acid, after which the cysteine in the hydrolysate was determined by oxidation with a selective oxidizing agent, phosphotungstic acid being used in our experiments. Control experiments showed, however, that when small amounts of cysteine, comparable to the amounts which the proteins were thought to contain, were added to the intact samples and the mixtures were subjected to digestion with the acid the added cysteine was only incompletely recovered. In one experiment in which the digestion was carried out in the presence of air, as some workers have done, the added cysteine vanished entirely.

The object of this paper is to demonstrate the inadequacy of the acid hydrolysis method for the determination of cysteine, and to report on studies of some of the factors responsible for the incomplete recovery.

EXPERIMENTAL

Except where otherwise noted, 100 mg. of protein (±10 per cent) were weighed out for the digestion. To this were added either 1, 2.5, or 5 mg. of cysteine (all ±10 per cent) as the hydrochloride. Two methods of hydrolysis were used. (1) The mixture of protein and added cysteine, contained in 2 ml. of 6 N HCl, was placed in a test-tube. To remove air, the tube was evacuated, then flushed with carbon dioxide which had been freed of oxygen by passing it over heated copper. This procedure was repeated several times. Finally, the tube was sealed off while evacuated and heated for 16 to 20 hours at 115°. (2) To the mixture of protein and added cysteine, contained in a 100 ml. acetylation flask, were added enough water to make the total amount of water 5 ml., 11 ml. of concentrated HCl, and 2 gm. of urea. The flask was attached to a reflux condenser and the system evacuated. Oxygen-free carbon dioxide was now admitted, and the pro-

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cedure repeated several times. A slow stream of carbon dioxide was allowed to pass through the system while the flask was heated in an oil bath to the point of gentle refluxing. The heating was continued for 6 hours.

This second procedure is essentially that of Brand and Kassell (1). Its purpose is to minimize formation of humin, an objective which they considered desirable in view of Lugg's (2) observation that cysteine reacts with humin. We have found it to be highly effective in preventing formation of insoluble matter. It gives perfectly clear and nearly colorless hydrolysates with horse serum albumin, ovalbumin, and lactoglobulin; the hydrolysate given by wool is faintly cloudy.

The acid was removed by vacuum distillation in a carbon dioxide atmosphere, and the residues were taken up in water and diluted to volume. The hydrolysates obtained by the sealed tube method were diluted to volume directly.

Cysteine was determined by the modified phosphotungstic acid method of Kassell and Brand (3).

Materials

Horse serum albumin[1] was prepared from lyophilized horse serum by the method of Adair and Robinson (4). It was recrystallized three times.

Ovalbumin was prepared from the whites of fresh eggs by the method of Sørensen and Høyrup (5). It was not recrystallized.

Lactoglobulin[1] was prepared from fresh milk by the method of Sørensen and Sørensen (6). It was recrystallized once.

The wool sample[1] had been scoured by the manufacturer in hot soap solution. It was further purified by extracting it successively with alcohol, ether, and water at room temperature.

Black human hair was purified by successive extraction with alcohol, ether, and water at room temperature.

Cysteine hydrochloride was obtained from the Eastman Kodak Company. Recrystallization did not affect its reducing power toward phosphotungstic acid.

Results

Table I shows the recoveries observed when cysteine was added to the proteins named above and the mixtures were submitted to the hydrolysis procedures outlined. It should be noted that these are "uncorrected" recoveries. They were calculated by subtracting, from the total amount of cysteine found, the amount contributed by the protein itself, as determined

[1] We should like to thank Sharp and Dohme, Inc., for supplying the lyophilized horse serum; Dr. W. G. Gordon of this Laboratory for lactoglobulin; Dr. Werner von Bergen, Forstmann Woolen Company, for the wool.

by a separate experiment in which no cysteine was added. This procedure can give accurate results only if the fraction of the cysteine lost is independent of the amount of cysteine present. Table I shows, however, that in all probability this is not the case, since the uncorrected recovery values show a strong dependence on the amount of cysteine, with 5 mg. additions giving much higher recoveries than the 1 mg. additions. It is

TABLE I

*Recovery of Cysteine in Acid Digestion of Mixture of Proteins and Added Cysteine**

Experiment No.	Protein	Cysteine added	Hydrolysis method	Per cent recovery of added cysteine†
		mg.		
1	Wool	1	Sealed tube	42.2
2	"	2.5	" "	57.5
3	"	5	" "	62.0
4	"	1	Urea	51.2
5	"	5	"	72.0
6	Human hair	1	Sealed tube	40.2
7	Ovalbumin	1	" "	59.0
8	"	2.5	" "	79.2
9	"	5	" "	63.5
10	Horse serum albumin	1	" "	31.9
11	" " "	2.5	" "	55.5
12	" " "	5	" "	70.0
13	" " "	1	Urea	33.3
14	" " "	5	"	73.5
15	Lactoglobulin	5	Sealed tube	81.6
16	"	5	Urea	79.2
17	None	5	Sealed tube	99.3
18	"	5	Urea	97.5

* The protein sample was approximately 100 mg. in each case. The terms "sealed tube" and "urea" refer, respectively, to the first and second methods of hydrolysis discussed under the heading "Experimental." The recovery value for the sealed tube control, Experiment 17, is the average of three, the individual values being 99.0, 99.4, 99.4 per cent. The recovery value for the urea control, Experiment 18, is the average of two, the individual values being 97.4 and 97.5 per cent.

† These are uncorrected recoveries; see the text.

therefore to be expected that the contribution of the protein itself will be larger in the recovery experiments than the values obtained in the absence of added cysteine. The recovery figures given in Table I are therefore probably too high.

The values found for the cysteine contents of these proteins, in the absence of added cysteine, are given in Table II. These, too, are "uncorrected," since it is not possible from the data at hand to say what is the proper correction factor to apply to them.

Examination of Table I shows that in no case could the added cysteine be completely recovered. The loss was particularly serious for the lower levels of added cysteine, although even at the 5 mg. level the loss was marked. Untreated proteins almost certainly contain much less than 5 per cent cysteine. Therefore, values obtained for these materials by acid hydrolysis methods may be appreciably low. Although the urea method is successful in preventing the formation of insoluble humin, Table I (Experiments 10 and 13, 12 and 14, 15 and 16) shows that it fails in three out of five cases to bring about any distinct improvement in cysteine recovery, compared with the sealed tube method. Moreover, Table II shows that it does not give higher values for the original cysteine content.

Two hypotheses suggest themselves to account for the missing cysteine: (1) It has reacted with residual oxygen, which has entered the system despite all precautions to exclude it; (2) it has reacted with some substance in

TABLE II

Cysteine Content of Proteins

Protein	Per cent cysteine*	
	Sealed tube method	Urea method
Wool	0.68	0.33
Human hair	0.42	
Ovalbumin	0.71	
Horse serum albumin	0.39	0.32
Lactoglobulin	1.01	1.01

* These values are on a moisture-free basis. They are uncorrected; see the text.

the protein sample, present either as an impurity or as part of the protein molecule.

The excellent recoveries of cysteine in the control experiments (Nos. 17 and 18, Table I) make the first hypothesis unlikely, since there was the same opportunity for oxygen to enter these systems as those containing protein. As a further check, a recovery experiment was performed on wool, identical with Experiment 5 in Table I, except that the quantities of wool and added cysteine were 5 times greater. If the loss of cysteine is due to reaction with a small amount of residual oxygen, then the fraction lost should be sharply decreased by increasing the amount of cysteine. Only a minor improvement in the recovery was observed, however, the value being 76.5 per cent.

The good recovery in the control samples also disposes of the possibility that the cysteine is decomposed in some fashion under the hydrolysis conditions, whether protein is present or not.

Concerning the second hypothesis, it is well known that cysteine can

react with reducing sugars and with aldehydes in general (see, for example, Schubert (7)). Many proteins contain firmly attached polysaccharide residues which are capable of producing simple sugars on digestion with acid. Many other protein preparations are likely to contain carbohydrate as an impurity. That glucose, at least, is capable of reacting with cysteine under the conditions ordinarily used for protein hydrolysis is proved by the data of Table III. These data were obtained as previously described for the protein samples. The results show that small amounts of glucose, such as might be produced by hydrolysis of the polysaccharide in 100 mg. of many protein samples, give rise to about the same cysteine losses as the protein samples in Table I.

Table III also shows that the urea modification of Brand and Kassell (1) materially diminishes the amount of cysteine lost during acid digestion with glucose. The digests yielded by the sealed tube method were strongly

TABLE III

*Recovery of Cysteine Digested with Glucose in Acid Solution**

Initial glucose	Per cent recovery of cysteine	
	Sealed tube method	Urea method
mg.		
2	78.3	90.8
5	60.8	84.4
10	41.5	72.5

* The initial amount of cysteine was 5 mg. in each experiment.

colored and contained appreciable residues of insoluble humin. The urea digests were much paler and were entirely free from sediment.

In view of the effect of carbohydrates on cysteine, three of the proteins used in this study, ovalbumin, horse serum albumin, and lactoglobulin, were tested for their carbonhydrate contents by the orcinol method of Sørensen and Haugaard (8). The results are given in Table IV. No attempt was made to determine the carbohydrate content of either wool or hair, owing to their insolubility. It has been shown, however, that wool, at least, contains a carbohydrate (10).

The values in Table IV do not necessarily indicate the total amounts of carbohydrate present, since glucosamine, which Neuberger (11) found in the polysaccharide of ovalbumin, does not respond to the orcinol test. The carbohydrate of horse serum albumin also appears to contain glucosamine (12). Sørensen (13) has shown that lactoglobulin contains not more than about 0.1 per cent glucosamine, and it is probably safe to assume that lactoglobulin is carbohydrate-free.

It is apparent from a comparison of Tables I, III, and IV that reaction between cysteine and carbohydrate can account for a large part of the loss of cysteine observed for those proteins which contain carbohydrate. The evidence indicates, however, that carbohydrate is not alone responsible, since we should then expect to observe a very marked improvement in recovery by the urea method, compared with the sealed tube method, by analogy with the results of Table III. The improvements in recovery revealed in Table I are much smaller than we should expect if the loss is all due to carbohydrate.

The best evidence that some protein component besides carbohydrate is involved is afforded by the results on lactoglobulin. Added cysteine is far from completely recoverable, although it is true that lactoglobulin gives the best recoveries of all the proteins investigated.

TABLE IV

Carbohydrate Content of Ovalbumin, Horse Serum Albumin, and Lactoglobulin; Orcinol Method

Protein	Per cent carbohydrate*
Ovalbumin	2.32
Horse serum albumin	1.27
Lactoglobulin	0.02

* These values are given in terms of a mixture of equal parts of glucose and mannose, according to Kekwick (9). They are on a moisture-free basis.

Brand and coworkers (14) have quantitatively specified the composition of lactoglobulin in terms of known amino acid residues. To test the possibility that cysteine reacts with other amino acids during the digestion process, a "synthetic lactoglobulin" was prepared by mixing all the amino acids except cysteine in the proportions given by Brand. To 116 mg. of this mixture, equivalent to 100 mg. of protein, were added 5 mg. of cysteine and 2 ml. of 6 N HCl. Digestion was carried out by the sealed tube method. Recovery of cysteine was 95.7 per cent. Thus, the loss of cysteine, although probably significant, is small, and one must conclude that there is little reaction between cysteine and the other amino acids in lactoglobulin.

The next hypothesis tested was that the cysteine reacts with some peptide, although it is incapable of reacting with the free amino acids. If this were the case, cysteine added to an already hydrolyzed sample of lactoglobulin should be recoverable to the same degree as the cysteine added to the synthetic amino acid mixture. 100 mg. of lactoglobulin were therefore hydrolyzed by the sealed tube technique. To the hydrolysate were added 5 mg. of cysteine, the air was displaced by carbon dioxide, and the tube was

resealed while being evacuated, and heated for another 16 hours at 115°. The recovery of cysteine was 81.2 per cent, in excellent agreement with the value of 81.6 per cent obtained when the cysteine was added to the intact protein (Experiment 15, Table I). It is evident that the loss of cysteine is not attributable to reaction with a peptide. This experiment also proves that the substance responsible for the loss is not destroyed by the digestion process.

Another possibility is that there is some impurity in the lactoglobulin sample which is not a carbohydrate but is capable of reacting with cysteine. A test of this hypothesis was made by performing a recovery experiment by the sealed tube technique on a sample of lactoglobulin which had been recrystallized four times.[2] A recovery of 84.8 per cent was obtained. This, although significantly higher than the 81.6 per cent recovery found for the once recrystallized material, still indicates a considerable loss of cysteine.

TABLE V

Recovery of Cysteine Added to Protein Hydrolysates

Protein*	Atmosphere during protein hydrolysis	Temperature during digestion with cysteine	Per cent recovery of cysteine
Horse serum albumin	Carbon dioxide	Boiling point	75.0
" " "	Air	" "	75.7
	"	Room temperature	76.9
Wool	"	Boiling point	74.4
"			

* The weight of each sample was 100 mg. 5 mg. of cysteine were added to each.

Thus, if some unidentified impurity which reacts with the cysteine is responsible, it is very difficult to separate this material from the protein by repeated crystallization. The possibility that it is not an impurity, but some as yet unrecognized part of the molecule, is not to be ignored.

As in the case of lactoglobulin, tests were made on horse serum albumin and wool to determine whether the recovery of cysteine added after hydrolysis of the protein is different from the recovery of cysteine added to the intact protein. These determinations, reported in Table V, were made by the urea method. To determine whether the hypothetical substance which reacts with the cysteine is stable to air oxidation, an experiment was performed on horse serum albumin in which the hydrolysis of the protein which preceded the addition of cysteine was carried out in one case in air and, in the other, under carbon dioxide. The subsequent digestion with the cysteine was performed under carbon dioxide in every case. To determine whether the reaction which leads to the loss of cysteine takes place at room

[2] We should like to thank Dr. R. C. Warner of this Laboratory for this sample.

temperature as well as at the boiling point, one experiment was carried out on wool, in which the mixture of wool hydrolysate and added cysteine was allowed to stand for 24 hours at room temperature. In the other experiments, the mixture of hydrolysate and added cysteine was refluxed for 6 hours.

Comparison of Table V with Table I confirms the conclusion previously reached for lactoglobulin; namely, that it makes little difference whether the cysteine is added to the protein before or after the latter is hydrolyzed. The interfering substance resists the action of acids, and is stable toward air oxidation. The reaction with cysteine takes place nearly to the same extent at room temperature as at the boiling point of the digestion mixture. Dialysis of a hydrolyzed lactoglobulin sample showed that the interfering substance passes through a cellophane dialyzing membrane.

DISCUSSION

The chief conclusion to which this study leads is that the determination of cysteine in a protein by a method involving acid hydrolysis is subject to serious error, an error, moreover, which it is difficult to determine quantitatively. In many analytical procedures which involve small losses, it is a common practice to add small amounts of the constituent sought, submit the mixture to the analytical procedure, calculate the per cent recovery of the added substance, and use this recovery factor to correct the value obtained for the desired constituent in the absence of any additions. This is especially true of samples of biological origin. Such a procedure is feasible, however, only when the correction is small (15 per cent is probably about the upper limit) and reasonably constant. In this case, however, the corrections are so large and so dependent on the amount of the desired constituent that no reliable correction seems possible.

It has been shown that only part of the loss can be attributed to reaction with carbohydrates. Part seems to be due to an unknown non-carbohydrate compound. The possibility that the unknown compound is an aldehyde was considered, but the fuchsin test applied to hydrolyzed lactoglobulin was negative. It is not claimed that the incomplete recovery shown by carbohydrate-free lactoglobulin proves the existence of an unidentified component of the molecule. It is conceivable that lactoglobulin actually is composed of nothing but known amino acids but that, during hydrolysis, small amounts of compounds other than amino acids are produced, compounds the formation of which depends on the spatial arrangement of the amino acids in the structure and which are, therefore, not produced by the digestion of a mixture of free amino acids.

If cysteine is lost during protein hydrolysis, as Tables I and V indicate, it

is necessary to explain the fact that for a number of proteins the determined amounts of cysteine, cystine, and methionine account satisfactorily for the total protein sulfur (1, 15–19). Most of these proteins, however, either contain no cysteine, or the contribution of cysteine to the total sulfur is so small that an error of 20 per cent in the cysteine estimation would not significantly affect the total sulfur. In the above references, of seventeen protein samples for which total sulfur is compared with the sum of cysteine, cystine, and methionine sulfur, there are only four for which correction of the cysteine content for an assumed 20 per cent loss disturbs the sulfur balance to any considerable degree. These are lactoglobulin (18), chymotrypsinogen (1), egg albumin (19), and myosin (19).

It may be that the lost cysteine is converted wholly or in part to cystine. This would make it possible for the total sulfur to be correct in spite of the fact that the cysteine is too low, since the sum of cysteine and cystine would be constant. Several experiments were performed by the authors to determine whether, in some of the experiments shown in Table I, the missing cysteine could be accounted for as cystine. The results were inconclusive, owing to the difficulty of determining a small change in cystine content of a solution containing relatively large amounts of both cystine and cysteine.

Regardless of the results of sulfur balance determinations, it is obvious that values for cysteine obtained by acid hydrolysis methods must be looked on with grave suspicion unless the method can be so modified as to give complete recoveries of added cysteine.

In view of the difficulties involved in acid hydrolysis, it may be best to seek some method for determining sulfhydryl which utilizes the intact protein. A beginning toward such a method has been made by Todrick and Walker (20), Kuhn and Desnuelle (21), and Mirsky and Anson (22).

SUMMARY

1. Cysteine added to proteins cannot be completely recovered after the protein is hydrolyzed by acid digestion, making doubtful cysteine determinations by this method.

2. Hydrolysis in the presence of urea prevents insoluble humin formation but only slightly improves the recovery of added cysteine.

3. Approximately the same loss of cysteine occurs if it is digested with a prehydrolyzed rather than an intact protein.

4. The presence of carbohydrate in some of the protein samples accounts partially but not completely for the loss of cysteine.

5. Lactoglobulin, a carbohydrate-free protein, reacts with cysteine during acid digestion, but a supposedly equivalent mixture of free amino acids does not.

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